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First cytogenetic information for *Drymoreomys albimaculatus* (Rodentia, Cricetidae), a recently described genus from Brazilian Atlantic Forest

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Abstract

The recently described taxon *Drymoreomys albimaculatus* is endemic to the Brazilian Atlantic Forest and its biology and genetics are still poorly known. Herein, we present, for the first time, the karyotype of the species using classical and molecular cytogenetics, which showed 2n=62, FN=62, and interstitial telomeric signals at the sex chromosomes. Nuclear and mitochondrial DNA sequences from the two karyotyped individuals verify the taxonomic identity as the recently described *D. albimaculatus* and confirm the relationship of the species with other Oryzomyini. Additionally, external morphological information is provided.

Keywords

Oryzomyini, karyotype, CBG banding, GTG banding, FISH, IRBP, Cyt *b*

Introduction

The Atlantic Forest harbors a high diversity of mammals, 20 percent of which are rodents of the subfamily Sigmodontinae (Ribeiro et al. 2009). However, the fauna of this biome is still barely known, such that discovery of new species is still common (De

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Vivo et al. 2010). Since 1999, 14 new species of sigmodontines were formally described for Atlantic Forest: *Abrawayaomys chebezi* (Pardiñas et al. 2009), *Akodon paranaensis* (Christoff et al. 2000), *A. philipmeyersi* (Pardiñas et al. 2005), *A. reigi* (González et al. 1999), *Brucepattersonius paradisus*, *B. guarani*, *B. misionensis* (Mares and Braun 2000), *Cerradomys langguthi*, *C. vivoi* (Percequillo et al. 2008), *Hylaeamys seuanezi* (Weksler et al. 1999), *Juliomys rimofrons* (Oliveira and Bonvicino 2002), *J. ossitenius* (Costa et al. 2007), *Rhipidomys tribei*, and *R. itoan* (Costa et al. 2011).

Recently, Percequillo et al. (2011) described *Drymoreomys albimaculatus* as a new monotypic genus, endemic to the Brazilian Atlantic Forest and known from a few localities in São Paulo and Santa Catarina states. Phylogenetic analyses based on morphological traits and DNA sequences [1143bp of cytochrome *b* (Cyt *b*) and 1235bp of interphotoreceptor retinoid binding protein (IRBP) genes] revealed the placement of *D. albimaculatus* in the tribe Oryzomyini, raising to 30 the number of extant Oryzomyini genera. According to those analyses, Percequillo et al. (2011) revealed that *D. albimaculatus* is the sister species of the Andean rat *Eremoryzomys polius*.

Here, we describe the karyotype of *D. albimaculatus* for the first time. In order to investigate the molecular identification of the two karyotyped animals, we added its Cyt *b* and IRBP sequences to the molecular data published by Percequillo et al. (2011). Additionally, we present morphological comments on the specimens.

Material and methods

Sampling

One male and one female were collected with pitfall traps in Santa Virgínia, Parque Estadual da Serra do Mar [45°03.00' to 45°11.00'W (DDM); 23°24.00' to 23°17.00'S (DDM)], state of São Paulo, Brazil. Pelage color and external measurements were taken during the fieldwork. Vouchers of both individuals are deposited in the Coleção de Mamíferos da Universidade Federal do Espírito Santo (UFES) under the catalog numbers UFES 2271 and UFES 2272.

Cytogenetic analyses

Metaphases were obtained *in vivo* from spleen and bone marrow, according to Ford and Hamerton (1956) with modifications. Conventional Giemsa staining was used to determine the diploid (2n) and the number of autosome arms (FN). GTG and CBG-banding were performed according to Seabright (1971) and Sumner (1972), respectively, with modifications. Fluorescent *in situ* hybridization (FISH) with a FITC labeled $(C_3TA_2)_n$ peptide nucleic acid (PNA) probe (DAKO) was carried out following the recommended protocol (Telomere PNA FISH Kit/FITC, Code No. K5325, DAKO). Mitotic plates were digitally captured with visible light or blue and green

filters (emission at 461 and 517 nm, respectively) in an Axioskop 40 epifluorescence microscope (Carl Zeiss) equipped with an Axiocam camera and AxionVision software. Images were overlaid and contrast enhanced with Adobe Photoshop CS5.1.

DNA extraction, amplification, and sequencing

DNA was extracted from liver with Chelex 5% (Bio-Rad) following Walsh et al. (1991). Amplification of an 820 bp fragment of Cyt *b* and a 782 bp of IRBP was performed with PCR using primers MVZ5 and MVZ16 (Irwin et al. 1991; Smith and Patton 1993), and A1 and F (Stanhope et al. 1992), respectively. Both extraction and PCR controls were used for each amplification. Each PCR mixture had 30 ng of DNA, 25 pmol of each primer, 0.2 mM of dNTP, and 2.52 μL of reaction buffer (50 mM KCl, 2.5 mM MgCl2, 10 mM Tris-HCl; pH 8.8), and 0.2 units of Taq DNA polymerase (Invitrogen) were added to complete 18 μL. Forty amplification cycles were performed in a thermal cycler (Eppendorf Mastercycler ep Gradient, Model 5341). Each cycle consisted of denaturation at 94°C for 30 s, annealing at 48°C for 45 s, and extension at 72°C for 45 s for Cyt *b*, and denaturation at 94°C for 30 s, annealing at 60°C for 60 s, and extension at 72°C for 180 s for IRBP. A final extension at 72°C for 5 min was performed for both Cyt *b* and IRBP amplifications. The PCR products were separated using 1% agarose gels in TAE buffer. Nucleotide sequencing was conducted using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Sequences of each animal were aligned with sequences from previously published data deposited on GenBank by Bonvicino and Moreira (2001), Weksler (2003), and Percequillo et al. (2011) using MAFFT ver. 5 (Katoh et al. 2005) under the iterative method of global pairwise alignment (G-INS-i). Our sequences were submitted to GenBank under accession numbers KF031014-KF031017.

Phylogenetic analyses

We performed maximum likelihood (ML) and Bayesian analyses using concatenated Cyt *b*-IRBP data set. For both analyses we used gene-specific unlinked models. The best-fitting model of nucleotide substitution for each gene was selected using the Akaike information criterion in accordance with the procedure outlined by Posada and Buckley (2004), and implemented in jModelTest, version 0.1.1 (Posada 2008). The maximum-likelihood trees were calculated using RAxML (Stamatakis 2006). The statistical support for the nodes was estimated by the nonparametric bootstrap, with 1000 pseudoreplicates (Felsenstein 1985). Bayesian analysis was performed using MrBayes 3.04b (Ronquist and Huelsenbeck 2003). Markov chains were started from a random tree and run for 1.0×10^7 generations, sampling every 1000th generation. The stationary phase was checked following Nylander et al. (2004). Sample points prior to the plateau phase were discarded as burn-in, and the remaining trees were combined to

find the maximum *a posteriori* probability estimated of the phylogeny. Branch support was estimated by Bayesian posterior probabilities (BPP). Two simultaneous analyses were performed to ensure convergence on topologies.

Results

Cytogenetic analyses

The animals showed 2n=62, FN=62, and the autosome set composed of 29 acrocentric pairs decreasing in size, and one small metacentric pair (Fig. 1A). The X is a large submetacentric, and the Y is a large submetacentric slightly smaller than the X (Fig. 1A). CBG-banding revealed pericentromeric constitutive heterochromatic blocks in all autosomes and in the long arm of Y (Fig. 1B). GTG-banding allowed the identification of almost all autosomic pairs, the X chromosome exhibited two interstitial bands at the long arm while a conspicuous pattern in the Y was not found (Fig. 1C). FISH detected telomeric signals at the ends of all chromosomes and additional telomeric sequences were found in the pericentromeric region of both X and Y chromosomes (Fig. 1D).

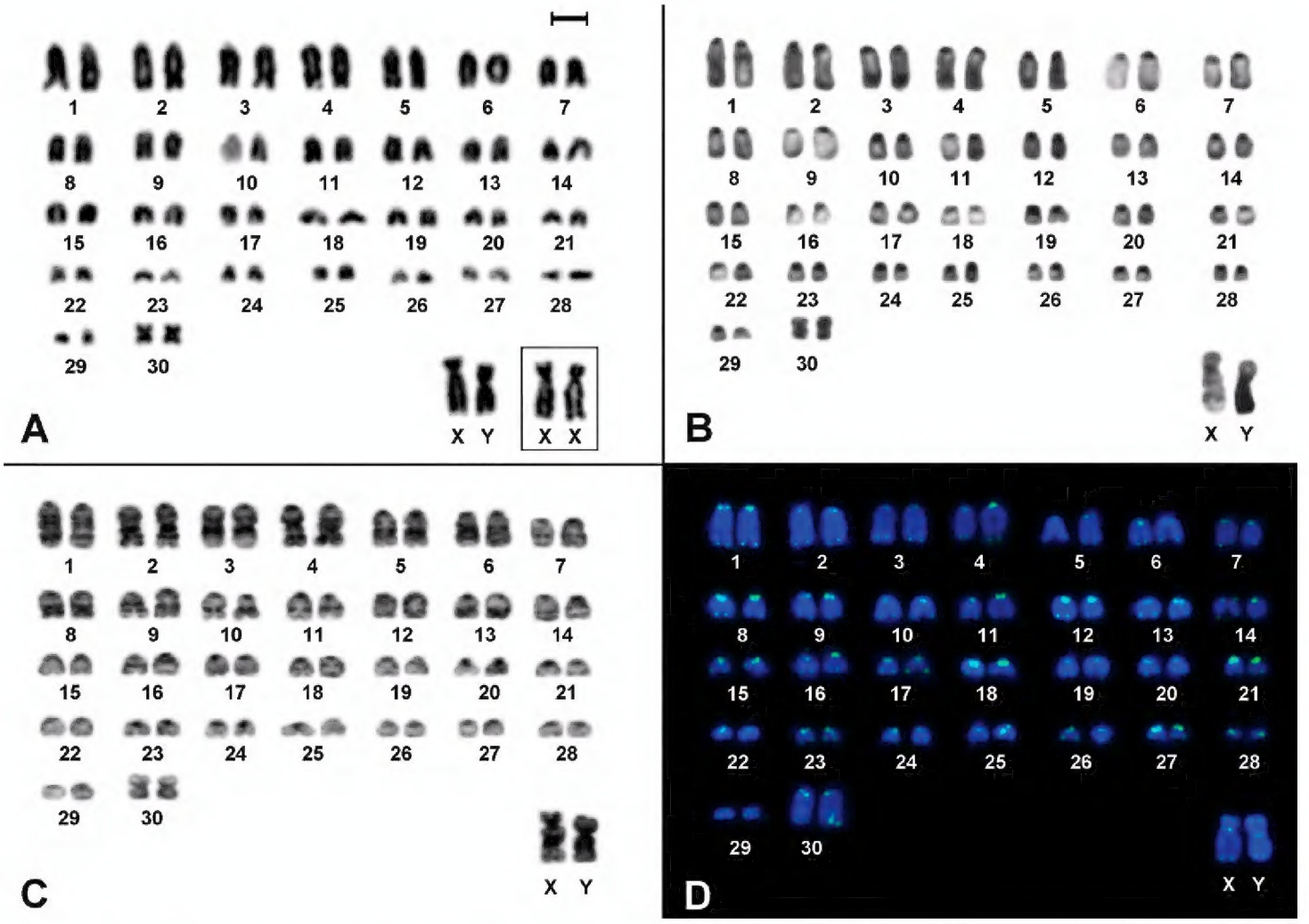

Figure 1. Cytogenetic analyses in *Drymoreomys albimaculatus* from Santa Virgínia, state of São Paulo, Brazil. **A** Karyotype of male (2n=62, FN=62), after conventional staining. Inset: sex chromosomes of a female **B** CBG-banding of a male **C** GTG-banding of a male **D** Fluorescent *in situ* hybridization using telomeric PNA probe over male mitotic plates. Bar scale = 10 μm.

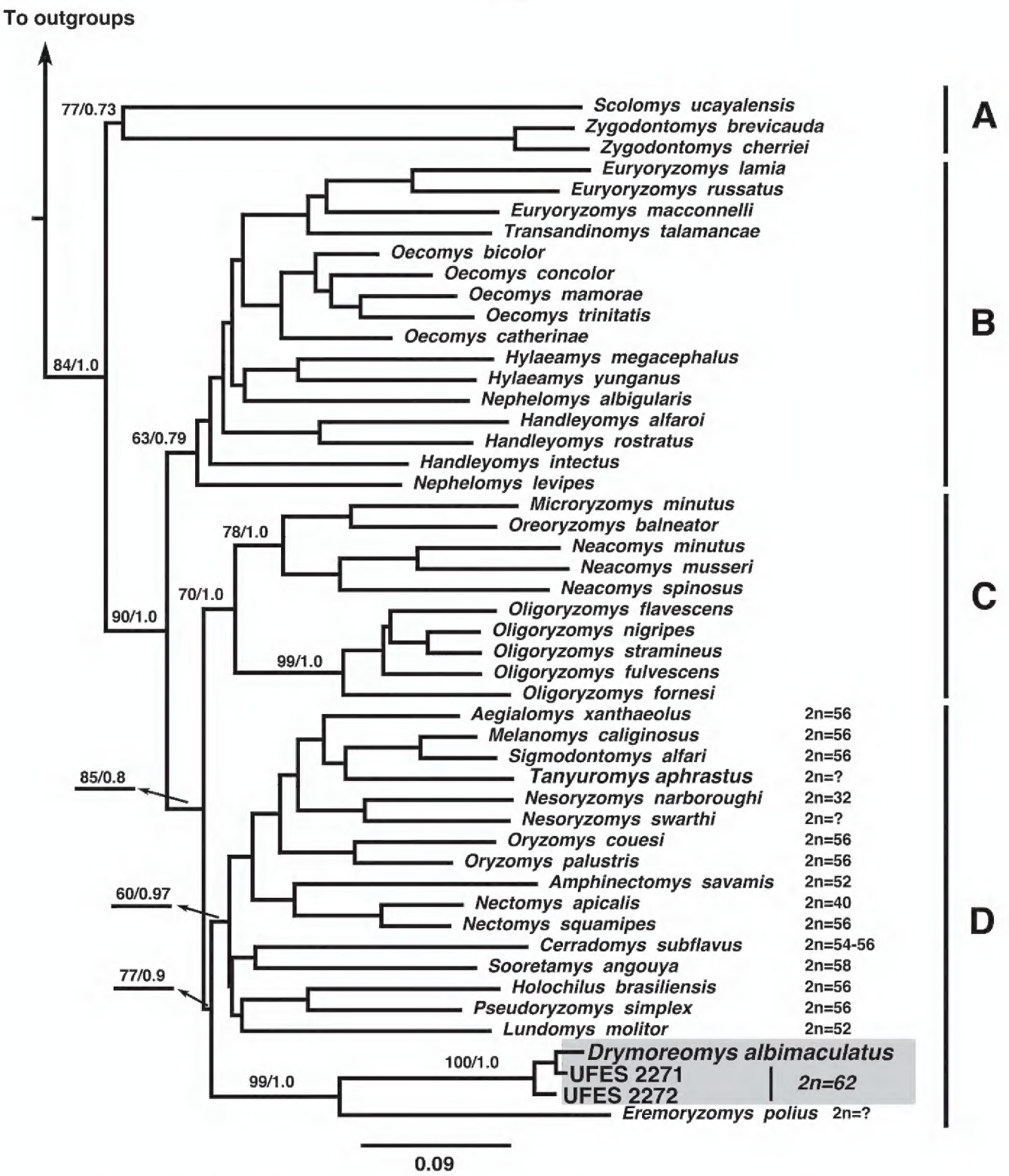

Figure 2. Maximum likelihood tree of combined molecular datasets [cytochrome b (Cyt *b*), interphotoreceptor retinoid binding protein (IRBP)] using Santa Virgínia specimens (UFES2271, UFES2272). Bootstrap nodal support indices and Bayesian posterior probabilities are shown above the branches, respectively. Outgroups include *Peromyscus maniculatus* (Neotominae); *Nyctomys sumichrasti* (Tylomyinae), *Delomys sublineatus* (Sigmodontinae), *Thomasomys baeops* (Sigmodontinae), and *Wiedomys pyrrhorhinos* (Sigmodontinae). Available diploid numbers (2n) of clade D are indicated (for details see Table 1), although the lowest diploid number (*Nectomys palmipes*, 2n= 16, Barros et al. 1992) does not appear in the figure.

Phylogenetic analyses

The model selected for the phylogenetic analyses (ML and Bayesian) was GTR +I + Γ for each gene. The best ML tree had a -ln likelihood score of -22,345.02. The Bayesian analysis recovered a consensus topology similar to the best ML tree and the results recovered the four well-supported clades A, B, C, and D (Fig. 2) previously reported

by Weksler (2006) and Percequillo et al. (2011). In both phylogenetic analyses, Santa Virgínia specimens (UFES 2271 and UFES 2272) clustered with high statistical support to the recently described *D. albimaculatus* (Fig. 2, grey area).

Discussion

Phylogenetic analyses (ML and Bayesian) recovered the four clades A, B, C, and D (Fig. 2) recovered by Weksler (2006) and Percequillo et al. (2011). In both phylogenetic reconstructions, Santa Virgínia specimens were recovered with high statistical support in clade D, confirming their identity as *D. albimaculatus* (Fig. 2, grey area), and consistent with Percequillo et al. (2011). Our analyses also recovered *D. albimaculatus* as the sister species of *Eremoryzomys polius* and both species diverged early in the clade D (Fig. 2).

The diploid number of *D. albimaculatus* corroborates the pattern found for the majority of the Oryzomyini species, in which karyotypes present relatively high chromosome number and predominantly acrocentric pairs. The typical heterochromatic pattern of sex chromosomes is also found in most of the oryzomyine species and it is an essential condition for the recognition of the Y (Fig. 1B).

The karyotype herein reported for *D. albimaculatus* is species-specific, since only three other Oryzomyini species present the same diploid number, but different FN: *Oligoryzomys fornesi* (2n=62, FN=64), *Oligoryzomys delicatus* (2n=62, FN=74 and 76), and *Oligoryzomys nigripes* (2n=62, FN=80, 81 and 82) (Gardner and Patton 1976; Weksler and Bonvicino 2005). Telomeric sequences at the pericentromeric region of *D. albimaculatus'* sex chromosomes could be hypothesized as (i) similar to regular sequences of the centromeres, (ii) related to a amplification of $(T_2AG_3)_n$-like satellite DNA repeats, or (iii) resulted of a structural rearrangement. In fact, interstitial telomeric sequences are common in vertebrates (Meyne et al. 1990) and apparently are a structural component of mammalian satellite DNA (Garagna et al. 1997; Pagnozzi et al. 2000). Additionally, these sequences have been associated with chromosome rearrangements (Ruiz-Herrera et al. 2008; Bolzan 2012).

A compilation of karyological studies in representative species of clade D is presented in Table 1. Notably, cytogenetic data in Oryzomyini has increased considerably in the last decades, mainly because the karyotype has become a valid tool for identifying species of this group. Although several species still remain without karyotypic information (e.g., *Eremoryzomys polius, Tanyuromys aphrastus, Nesoryzomys swarthy*), the diploid number within clade D varies from 16 in *Nectomys palmipes* (Barros et al. 1992) to 62 in *D. albimaculatus*. As *D. albimaculatus* exhibited the highest diploid number reported hitherto and diverged early in clade D, karyotype evolution in this clade based on the phylogeny (Fig. 2), apparently exhibits a trend toward a decrease in the diploid number. This hypothesis could imply chromosomal plasticity in low 2n ratios as suggested by Gardner and Patton (1976). In this sense, tandem fusions have perhaps played significant role in clade D, resulting in the lower diploid numbers. Robersonian rearrangements could have occurred in this group as well, since some species of clade

Table 1. Cytogenetic characteristics of Oryzomyini species of clade D, with diploid number (2n), fundamental number (FN), morphologies of autosomal pairs and sex chromosomes, polymorphisms described and references. *Supernumerary chromosomes are not included in autosomal morphologies. A= acrocentric; M=metacentric; SM=submetacentric; ST=subtelocentric; ITS = interstitial telomeric signals; NA= not available.

Species	2n	FN	Autosomal morphologies*	Sex chromosome morphologies	Cytogenetic characteristics	References
Aegialomys xanthaeolus	56	58	25 A 2 M/SM	X: large A Y: small A		Gardner and Patton (1976)
Melanomys caliginosus	56	58	25 A 2 M	X: large ST Y: medium ST		Gardner and Patton (1976)
Sigmodontomys alfari	56	54	27 A	X: large A Y: small A		Gardner and Patton (1976)
Tanyuromys aphrastus	NA	NA	NA	NA		
Nesoryzomys narboroughi	32	50	5 A 8 M/SM 2 ST	X: medium A Y: small A		Gardner and Patton (1976)
Nesoryzomys swarthi	NA	NA	NA	NA		
Oryzomys couesi	56	56	26 A 1 M	X: large SM Y: medium A/ST	Y heteromorphisms	Haiduk et al. (1979)
Oryzomys palustris	56	56	26 A 1 M	X: large A Y: minute A		Haiduk et al. (1979); Gardner and Patton (1976)
Amphinectomys savamis	52	66	NA	NA		Malygin et al. (1994) apud Musser and Carleton (2005)
Nectomys apicalis	42	40	20 A	X and Y: A		Patton et al. (2000)
Nectomys squamipes	56–59	56	26 A 1 M	X: large SM/ ST Y: medium/ small SM/ST	0-2 B chromosomes; sex chromosomes polymorphisms	Maia et al. (1984)
Cerradomys subflavus	54–56	62	21 A, 3 SM, 2M 23 A, 2 SM, 2M	X: large A/ ST Y: medium A/ large A	Centric fusion/fission, pericentric inversion, sex chromosomes polymorphisms	Almeida and Yonenaga-Yassuda (1985)
Sooretamys angouya	58, 60	60, 64	26 A 2 M	X: large A Y: medium ST	0 or 2 B chromosomes	Andrades-Miranda et al. (2001); Silva and Yonenaga-Yassuda (2004)
Holochilus brasiliensis	56–58	56, 58, 60	26 A 1 M	X: large ST Y: small SM	0 to 2 B chromosomes	Yonenaga-Yassuda et al. (1987)
Pseudoryzomys simplex	56	54, 55	27 A	X: large A Y: medium A	Heteromorphic pair 17 due to addition of constitutive heterochromatin	Voss and Myers (1991); Moreira et al. (in press)
Lundomys molitor	52	58	21 A 4 M	X: large SM Y: small M	X heteromorphism	Freitas et al. (1983)
Drymoreomys albimaculatus	62	62	29 A 1M	X: large SM Y: medium SM	ITS in both sex chromosomes	Present study
Eremoryzomys polius	NA	NA	NA	NA	NA	

D present the same FN but different 2n and number of biarmed chromosomes (e.g., *D. albimaculatus* and *Cerradomys subflavus*, Table 1). Non-Robertsonian mechanisms such as pericentric inversions, unequal translocations, or whole-arm heterochromatin addition or deletion could also be invoked in those cases of changes in FN but not in 2n (e.g., *Sigmodontomys alfari*, and *Melanomys caliginous,* Table 1).

Species of clade D present sex and supernumerary chromosomes easily identifiable with classical cytogenetic approaches, and some species exhibit sex chromosomes with polymorphisms/heteromorphisms and interstitial telomeric signals (ITS; Table 1, Fig. 1D). Thus, this clade is an excellent model to study origin, evolution, and chromatin composition of these chromosomes. For instance, a superficial morphological comparison among sex chromosomes from Table 1 could suggest the occurrence of pericentric inversions, or whole-arm heterochromatin additions or deletions.

Comments on external morphology and natural history

The specimens collected were medium sized (male body mass: 46.5 g, head and body length: 115 mm, and tail length: 142 mm; female body mass: 57 g, head and body length: 127 mm, and tail length: 170 mm). Tail was longer than head and body, and was a uniform color on both sides. Male hind foot was short (25 mm, 22% of head and body length) and ears were small (16 mm; 14% of head and body length). These external morphological measures overlapped with those of the *Drymoreomys albimaculatus* holotype (Percequillo et al. 2011). Dorsal pelage was reddish-brown; ventral pelage was predominantly grayish. Samples exhibited the pattern of short hind feet consistent with *Oecomys*. Fore and hind feet digits were covered by silvery-white hairs and the dorsal surface of hind feet were covered by brown hairs forming a patch, in a more conspicuous pattern than the observed for *Rhipidomys*. Thus, some external morphological traits were similar to those described for *Rhipidomys* and *Oecomys* as reported Percequillo et al. (2011). Nevertheless, our samples exhibited the characteristics of the *D. albimaculatus* holotype that differentiate it from *Rhipidomys,* such as the shorter, thinner, and sparser mystacial vibrissae and presence of gular to pectoral patches of white hair. Additionally, we detected that, contrary to what is found in *Rhipidomys,* a tuft of hairs on the tail's end is absent in our samples. On the other hand, several anatomical traits that distinguish the *Drymoreomys albimaculatus* holotype and species of *Oecomys* were observed in our samples, such as the plantar surface of pes covered with squamae; dorsal surface of pes with dark patches of brown hairs and the ventral pelage with gular and thoracic white patches (Percequillo et al. 2011).

Percequillo et al. (2011) reported that most of the *Drymoreomys* specimens were collected in pitfall traps; in the present work, the animals were also collected in the same way. These reiterate the importance of further fieldwork effort, with different collecting methods in order to increase the spectrum of small mammals collected. Consequently, our knowledge of small mammal biodiversity will be improved as a whole, which will allow improvements in relevant laws and policies for biodiversity protection.

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